Methionine Restriction Increases Insulin Sensitivity in Type-2 Diabetes via miRNA Activation

Yuanming Li[1*], Zifu Yu[2], Tuowei Li[3], Chuanzi Li[4]

[1]Beijing City International School, Beijing, 100022, China
[2]The Williston Northampton School, Easthampton, 01027, USA
[3]Pennsylvania State University, State College, 16803, US
[4]Xi'an middle school of Shaanxi province, Xi'an, 710000, China

Abstract. Methionine Restriction (MR) causes a higher level of circulating and hepatic fibroblastic growth factor 21 (FGF21). This leads to metabolic phenotypes, including increased energy expenditure, insulin sensitivity, and extended lifespan. Previous studies on obese mice have concluded that dietary MR in a high-fat regimen prevents hyperglycemia and improves glucose homeostasis, thus preventing type-2 diabetes, a multifactorial metabolic disease characterized by high blood glucose levels and cell insulin resistance. Recent experiments have shown that cells' response to dietary MR includes changes in methylation of DNA promoters that activate or repress microRNAs (miRNAs), which are small endogenous nucleotide sequences and contain 18-22 base pairs that control gene expression for lipid metabolism. Considering that the disruption of miRNA levels affects insulin resistance, miRNA potentially plays a role in MR to increase insulin sensitivity for type-2 diabetes. In this paper, we investigate the mechanism of MR influencing the expression level of miRNA-15b to promote insulin sensitivity in obese organisms. Using our in-vitro model, we measured the expression of miRNA-15b in adipocytes cultured in MR and control conditions. Additionally, we compared insulin sensitivity and free fatty acid (FFA) metabolite levels between obese mice on control and MR diets. Taken together, we were able to verify the positive effects of MR in reducing hepatic fatty acid production, decreasing blood glucose levels, and increasing insulin sensitivity. However, miRNA-15b downregulates cells' insulin signaling pathway and insulin sensitivity. Therefore, we proposed potential influences of MR on other miRNAs in reducing lipid cell differentiation and enhancing insulin sensitivity for future investigation.

1. Introduction

1.1 Background

1.1.1 Type-2 diabetes.

Type-2 diabetes, the most common type of diabetes developed among adult Americans (90-95% of diabetic patients) in the US, is caused by a variety of risk factors, including genetic inheritance, a lack of physical activities, a diet with high dietary fatty acid and glycemic index [1], and other complications such as overweight. The onset of this chronic metabolic disorder is triggered by insulin resistance inside cells. When cells don't normally respond to insulin signals to uptake blood glucose, the b-cells in the pancreas secrete more insulin to facilitate cellular responses. However, insulin-resistant cells impede insulin regulation and eventually causes blood glucose level to rise in the bloodstream [2].

1.1.2 Insulin resistance.

Insulin resistance refers to "the cellular response to insulin is impaired and the normal amounts of insulin cannot achieve normal glucose homeostasis". It often correlates to errors in the insulin signaling pathway. In a functional insulin signaling pathway, the signaling process is initiated as insulin binds to the insulin receptor (INSR) on the surface of the cell. Then, the insulin receptor substrate launches a series of downstream signaling with receptors including "phosphoinositide 3-kinase (PI3K), AKT serine/threonine kinase (AKT), and glucose transporter 4 (GLUT4)" [3,4].

1.1.3 Methionine restriction.

Methionine restriction (MR) plays an important role in improving metabolic health. Methionine metabolism at molecular levels creates a master methyl donor called S-adenosylmethionine (SAM). Then, the methylation of cytosines in the genes' promoter regions can control the binding of transcription factors to regulate gene expression [5].

* Corresponding author. Email: liyuanming27@gmail.com

By changing methionine availability in dietary MR, the number of available methyl donors can be potentially reduced, which changes the transcriptional status of specific genes, such as fibroblast growth factor 21 (FGF21) [6]. MR induces elevated levels of FGF21 circulating in the plasma, which leads to metabolic responses such as higher energy expenditure (EE) and increased insulin sensitivity in the body. Based on data in previous experiments conducted on New Zealand obese (NZO) mice in a high-fat regimen, mice in the MR-fed group exhibit increased FGF21 mRNA expression levels, higher EE, and significant improvements in cellular insulin sensitivity compared to mice in the Con group after 9 wk. Though the complete underlying mechanism of dietary MR-induced interaction needs further exploration, it is suggested that dietary MR leads to elevated plasma FGF21 levels and protects NZO mice from the onset of hyperglycemia and type-2 diabetes.

1.1.4 Micro-RNA.

Recent articles have begun accessing the miRNA role in the effect of MR. Micro-RNAs (miRNA) are non-coding small 18-22 nucleotide sequences produced from double-stranded precursors, enzyme Drosha and enzyme Dicer, in the nucleus and cytoplasm. Before integration into the RNA-induced silencing complex (RISC), miRNAs are bound by Argonaute subfamily of protein. After the integration, miRNAs can suppress transcription and translation of target transcription factors through complementary base pairing. As induced by dietary MR, the changes in chromatin methylation or methylation status of DNA promoter methylation can activate or inhibit the microRNAs (miRNAs) involved in cells response to the restricted diet. As miRNA regulates a series of mRNAs to maintain homeostasis, it controls lipid metabolism gene expression. These obvious changes may be related to the phenotype of MR, which is characterized by an increase in longevity, glucose tolerance, and metabolic rate.

Previous studies have demonstrated the association between miRNA and Caloric restriction (CR), indicating that these regulatory RNA molecules are responsible for CR-induced longevity to some extent. In recent years, researchers began evaluating miRNA's role in similar MR situations. The experiments of Rainbow trout myosatellite cells showed that miRNA played a role in the MR phenotype of mammals and fish. When exposed to the methionine-deficient cell culture, the rainbow trout myosatellite cells would regulate miRNAs that reduce their ability to differentiate (miR-133a, miR-206, and miR-210). These results demonstrate the role of MR in changing the mammals' and fish's metabolic phenotype while indicating that miRNA is a potential mechanism of MR（see Figure 1）.

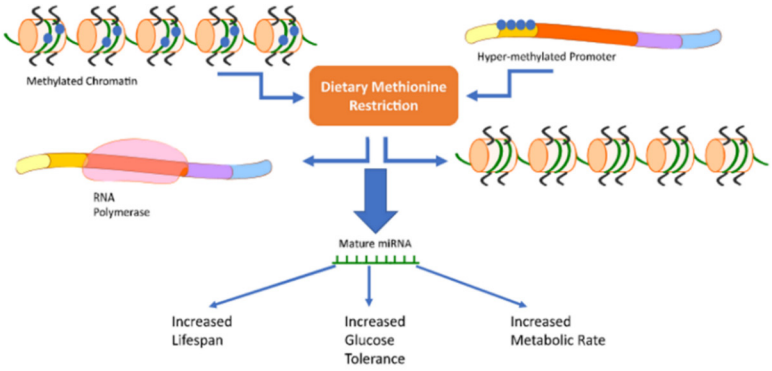

Fig. 1. Due to MR, changes in DNA promoter chromatin methylation or methylation status can activate or inhibit microRNAs involved in the cell's response. The varieties are characterized by increased lifespan, increased glucose tolerance, and increased metabolic rate and may be related to the MR phenotype [7].

1.2 Hypothesis

This experiment aims to test whether miRNA-15b, the regulator of the INSR gene, the first signal in insulin signaling, is included in the underlying mechanism of MR. It is speculated that miRNA-15b is induced by MR to promote insulin sensitivity preventing the onset of type 2 diabetes. If it holds, miRNA-15 should be successfully activated by MR and produce metabolites that improve insulin sensitivity in the cell. If it is false, then miRNA-15b should not be related to the MR's insulin sensitivity improvement. It might also negatively affect the production of metabolites and thus increase insulin resistance.

2 Methods and Results

2.1 MiRNA-15b expression level in methionine restriction

2.1.1 Steps.

1. testing the impact of methionine restriction on the expression level of miRNA-15b in order to test Methionine Restriction's relationship to miRNA-15b expression level. To test the direct impact, the approach will be cell culture. Adipocytes were cultured in the Dulbecco's modified eagle's medium (DMEM, ThermoFisher Gibco) with 10% fetal bovine serum and 1% penicillin-streptomycin as the growth media. After complete fusion, cells were transplanted into DMEM supplemented with 2% horse serum

and 1% penicillin-streptomycin. After 3 to 4 days of differentiation, used fresh serum-free DMEM (control group) or methionine-free DMEM to replace the medium (MR, ThermoFisher Gibco). Within 8 to 16 hours of incubation, the medium and cells were collected for the specified assay[8]. Then the quantitative real-time PCR method will be used to test whether the miRNA-15b expression level has been influenced.

2. testing the impact of methionine restriction on the function of miRNA-15b in vivo. Whether the expression level of miRNA-15b is influenced, the real impact of the methionine restriction on the miRNA-15b's function in INSR also needs to be tested. Type-2 diabetes models fat mice will be adopted for the experiment, dividing 40 mice into four groups with different conditions.

Table 1. 4 Type-2 diabetes model mice groups in comparative diet[Owner-draw]

Group 1 Mice will eat a high-calorie diet, which keeps the amount of methionine.	Group 2 Mice will eat a high-calorie diet, which decreases the amount of methionine in each diet.
Group 3 Mice will eat a high-calorie diet that keeps the amount of methionine and downregulates the miRNA-15b by using its anti-sense miRNA.	Group 4 Mice will eat a high-calorie diet, which decreases the amount of methionine in each diet and downregulate the miRNA-15b by using its anti-sense sequence miRNA.

Note：high calorie diet:protein, 16 kcal%; carbohydrate, 52 kcal%; fat, 32 kcal%)

The insulin sensitivity can be detected by testing the changing speed in glucose and insulin level in blood after diet（see table 1）.

2.1.2 Expected result.

The expression level in the two groups in step1 does not have an obvious difference. It revealed that methionine does not affect the level of miRNA-15b. In step 2, the mice in group 1 have a lower insulin sensitivity level than those in group 2. The mice in group4 have a higher insulin sensitivity than group 2, meaning that the methionine also does not affect the miRNA-15b's function on INSR, and the miRNA-15b level is negatively related to insulin sensitivity. The methionine restriction did not impact the expression level of miRNA-15b and did not influence the function of miRNA-15b in INSR. Alternatively, the miRNA-15b accompanies the downregulation of the function in INSR [4]. Therefore, the type-2 diabetes model will not be influenced by methionine restriction via affecting miRNA-15b. Furthermore, the abnormality of miRNA-15b can also be doubted as a factor supporting type-2 diabetes. The insulin resistance may be influenced by inhibiting the function of miRNA-15b in INSR, which may be applied to further research about type-2 diabetes.

2.2 Diet Control

Blood sugar refers to the concentration of glucose in the blood. Insulin is a hormone secreted by the beta cells of the pancreatic islets that lower blood sugar, and it is the only hormone in the body that can lower blood sugar. When blood sugar rises, promote insulin secretion is promoted. Insulin can promote excess glucose into glycogen and inhibit glycogen into glucose. Insulin sensitivity is related to the degree of insulin resistance. The blood sugar level is an indicator of insulin sensitivity. Under this background, research focuses on whether obese mice's blood sugar level is lower, meaning their insulin sensitivity is higher in a methionine restriction and a normal diet. A methionine restriction diet controls the intake of methionine, which means restricting the normal methionine intake by 20 percent.

2.2.1 Steps.

1. 20 New Zealand obese (NZO) mice are used to do this experiment with ten mice in each group: one group provides the MR diet, the other group provides a normal diet.

2. In the MR diet group, mice are provided normal food and methionine restriction food. In the control group, mice are provided a normal diet with other ammonia acids that do not influence the final blood sugar.

3. After nine weeks, OGTT is used to measure the blood sugar in each group.

OGTT Test

1. The definitive test for the diagnosis of diabetes.

2. Method refers to the oral administration of 75g of anhydrous glucose in an aqueous solution.

3. Blood samples were collected at 0.5, 1.0, 1.5, and 2.0 hours to measure the change in blood glucose.

4. Observe the ability of patients to tolerate glucose.

2.2.2 Result.

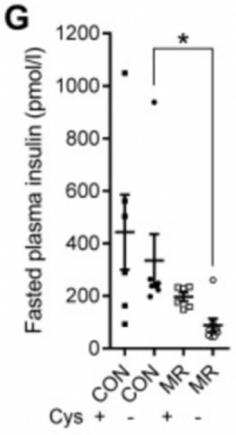

Fig. 2. Under the curve means insulin sensitivity [9]

At the end of the experimental intervention, the blood glucose levels of control group (Con) mice (15.5 ± 1.7 mM)

increased. The blood glucose levels of MR mice (7.4 ± 0.3 mM) were normally exhibited. According to Figure 2, fasting plasma insulin levels in control group (Con) mice (335.3 ± 100.8 pM) were higher than that in MR mice (88.1 ± 25.4 PM) [10].

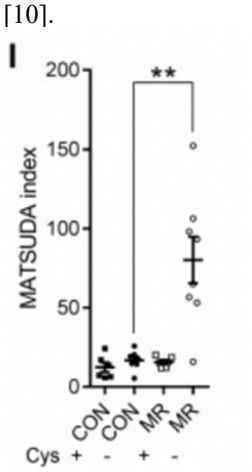

Fig. 3. Matsuda index is used to calculate insulin sensitivity [11]

Con-type diet mice contain higher blood sugar levels than the MR diet mice, indicated by higher insulin levels. From Figure 3, the Matsuda index shows an improvement in insulin sensitivity in MR mice (80.1 ± 14.6) compared with Con mice (16.8 ± 2.4) [12]. With lower blood sugar, MR diet mice have higher insulin sensitivity. Higher insulin level and blood sugar about the Con-type diet mice have higher blood sugar than the MR diet mice. Determine blood sugar: higher blood sugar, lower insulin sensitivity. According to the experiment, the research finds that the MR diet mice have lower blood sugar, which means they have higher insulin sensitivity.

2.3 Difference of Metabolites (FFA) between MR and T2D Group

Metabolites are the closest to biological phenotypes. Metabolomics focuses on the endogenous metabolites of organisms. These metabolites are obtained through sugar, fat, protein, nucleic acid, bile acid, symbiotic bacteria, and so on. Metabolomics aims to find potential differences and markers by comparing metabolic samples to carry out the early diagnosis of diseases and even research on gene and protein levels. It is possible to examine the changes in the composition and content of all small molecule metabolites (molecular weight less than 1500 Da) before and after stimulation or perturbation of a biological system in a specific period. Moreover, metabolomics can find the relative relationship between metabolites and physio-pathological changes.

2.3.1 Methods.

1. Animal experiments

Type-2 diabetic mice were modeled in the same way as before. Randomly divided twenty diabetic mice into the model group and the methionine restriction diet group. Each group contained 10 New Zealand obese mice. The model group was provided with standard feed every day. The low methionine diet group was provided feed containing only 0.86% methionine daily. Kept feeding mice.

2. Sample collection and preparation

Extracted the blood from the retroorbital venous plexus from the control and methionine restriction diet groups on week one, week two, week four, and week eight and centrifuged at 3000 r/min to collect the serum. Store the samples at -80 ℃.

3. Targeted Metabolomic analysis

Before sample detection, melted the serum samples. Detected amino acids (butanoate, glutamate, branched-chain amino acids, and purines) in blood by spectrophotometry. Used selective ion monitoring (SIM) in GC/MS system to test the levels of lipids and free fatty acids in serum from different groups. Detected free fatty acids by copper salt reaction and GC/MS system to find the absorption peak at 715 nm and used quantitative analysis software to determine lipocalin and free fatty acids.

4. Combined histological analysis

Used simultaneous metabolomic analysis of sera from mice in the diabetic group and KEGG analysis of differential metabolites to find particular amino acid contents, lipid export induction, and obesity-associated inflammatory responses.

2.3.2 Results.

The relative contents of butanoate, glutamate, branched-chain amino acids and purines in the model group were higher than that in the MR diet group [13]. The content of free fatty acids in the blood of mice in the MR diet group was lower than that in the model group. The control results showed that methionine restriction decreases fatty acid metabolism in T2D patients. Methionine restriction lowers blood glucose through non-insulin signaling-dependent pathways and stimulates insulin secretion, thereby lowering blood glucose.

2.3.3 Discussion.

Since the model could not be maintained in a diabetic state all the time as the mice ate a regular diet, they will naturally revert to a healthy state, and the metabolism of amino acids and fats in the mice should be tested at regular intervals. Insulin governs the level of blood glucose and is also the primary regulator of lipid and protein metabolism. Insulin resistance causes the overproduction of free fatty acids and defective clearance of LDL and triglycerides. Therefore, when the biological regulation of insulin is impaired in diabetic patients, it is often accompanied by abnormalities in the metabolic pathways of short-chain fatty acids and the problem of hyperlipidemia. As a result, fat accumulates in the form of lipid droplets in cells of other organs, which in some cases can cause lip toxicity. Some of the complications associated with T2DM result from FFA metabolic dysregulation. In addition, FFA is directly and indirectly associated with producing inflammatory molecules and regulating inflammation, common T2DM comorbidity. Lipid abnormalities may also affect cell membranes, which may impair cellular function. Fat is usually stored in adipocytes in the form of TAG, but when

plasma FFA levels are too high, adipocytes can become overwhelmed. FFA in plasma might mediate insulin resistance and impaired glucose tolerance associated with central obesity（see Figure 4）.

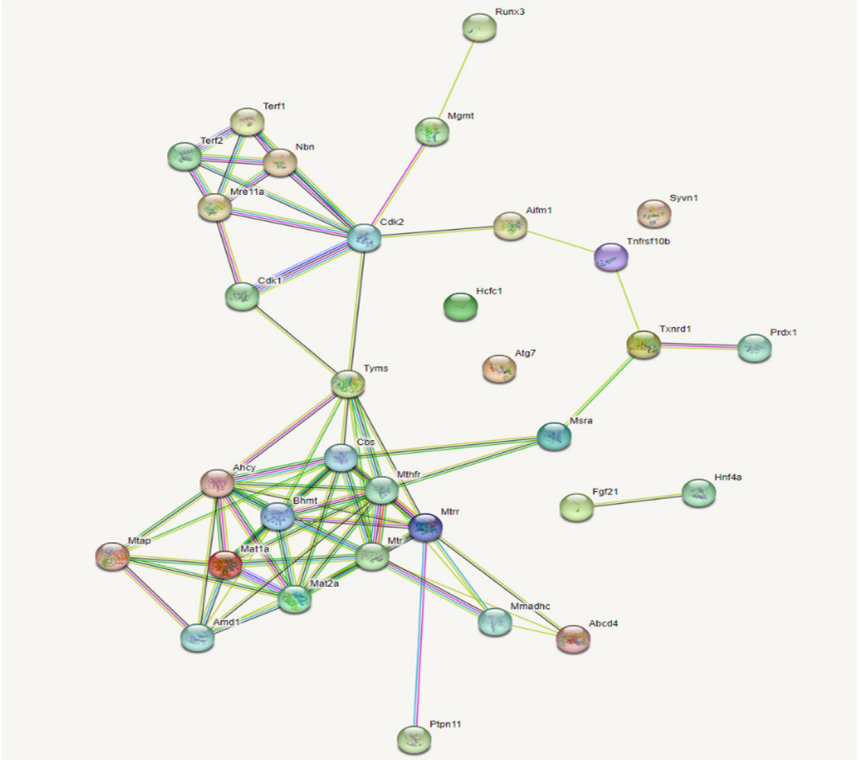

Fig. 4. Pathway targets related to methionine restriction and type II diabetes mellitus obtained by STRING [14]

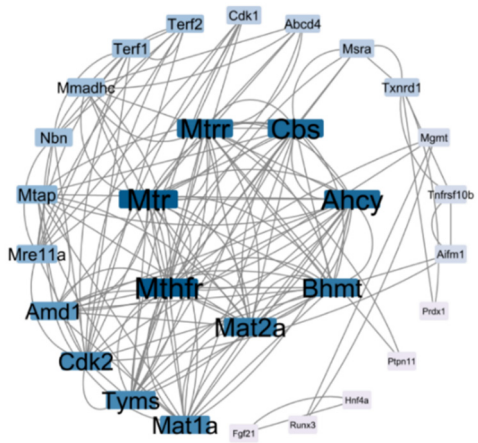

Fig. 5. In the protein interaction network diagram obtained by network topology, the higher degree the more important it is. The higher the degree, the darker the color and the larger the icon [15]

Due to the specificity of metabolomics, differences in the individual genes and protein regulation can be viewed by metabolites. If miRNAs are altered, differences in metabolites can be inferred. However, even though miRNA and MR pathways interoperate, more studies are needed to discover the association between the two with diabetes（see Figure 5）.

3 Conclusion

In the NZO mice diet control experiment, the MR group mice displayed a lower blood sugar level than the control group, demonstrating the effectiveness of MR in improving insulin sensitivity in cells. However, in the miRNA-15b experiment, MR shows no impact on the expression level of miRNA-15b, nor did the function of miRNA-15b in INSR. Conversely, miRNA-15b accompanies the downregulation of the function in INSR. Therefore, the T2D model will not be influenced by methionine restriction via affecting miRNA-15b, and the abnormality of miRNA-15b is also doubted as a factor causing T2D. Dietary methionine restriction has been reported to affect other microRNAs' abundance and improve metabolic phenotypes. Rainbow trout fed an MR diet have a lower miR-133a level in skeletal muscle cells after four weeks and increased glucose tolerance at eight weeks compared to rainbow trout receiving MS diets. MicroRNA-133a regulates the expression of transcription factors for myogenic precursor cell (MPC) differentiation status in mammals' muscles, which alters the lean muscle mass in muscle metabolic health. It is regulated by the myogenic factors Myogenin and MyoD1. As shown in previous in vitro studies, there was an increase in Myogenin and MyoD1 in normal differentiation and an associated reduction in Myogenin and MyoD1 during MR, which might induce a reduction in miR-133a signaling. To continue investigating the underlying mechanism of methionine restriction in increasing insulin sensitivity, we propose that the decrease in miR-133a expression level in MR leads to reduced muscle lipid accumulation and improved insulin sensitivity.

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